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RESEARCH ARTICLE

Ex-situ evaluations of *Lolium perenne* L. ecotypes collected in Bulgaria, Croatia, Spain and Ireland reveal valuable breeding material

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Abstract Between 2002 and 2010, a total of 366 *Lolium perenne* L. genebank accessions originating from collection trips to Bulgaria, Croatia, Spain and Ireland were evaluated in field experiments together with commercial check varieties at the island of Poel in northern Germany. Ten plant parameters were visually scored, including the development before and after winter, spring growth, heading, plant biomass and the incidence of disease symptoms. The ecotypes collected in Croatia and Spain achieved high scores for all the growth parameters evaluated, particularly in the first and second experimental year, and were well comparable to the check varieties. The Bulgarian ecotypes exhibited a low plant biomass development after the first cut and before winter but a high productivity in spring and a low susceptibility to rust infection. The ecotypes from Ireland revealed a low aftermath heading in the second experimental year and a superior growth after winter in the third year. In conclusion, the *L. perenne* ecotypes studied showed a high variability regarding important agronomic attributes and several accessions were identified that exceeded the average performance of the check varieties.

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Introduction

As one of the most frequent grassland species in temperate climates, perennial ryegrass (*Lolium perenne* L.) has been subject of intensive breeding and research. It gains special importance in forage production as it establishes rapidly, grows easily and achieves high yields and a good feeding value for ruminants (Easton et al. 2011; Sampoux et al. 2011; McDonagh et al. 2016). Agronomical traits of interest in forage grass breeding are herbage yield and quality, heading and aftermath heading, disease susceptibility, winter hardiness, persistence and, recently, tolerance to heat, drought and reduced fertilizer input. Furthermore, perennial ryegrass is the most widely sown turf grass in Europe and many other temperate regions of the world (Humphreys et al. 2010; Sampoux et al. 2013). Like forage varieties, turf varieties ought to have a good persistence, resistance to diseases and wear tolerance, but a limited growth and thin leaves (Romani et al. 2002).

So far, considerable success in perennial ryegrass breeding has been achieved in the past decades. As the most important trait for forage production, cumulative dry matter yield of perennial ryegrass cultivars increased between 0.3 and 0.9% annually during the past 40–50 years (Wilkins and Humphreys 2003; McDonagh et al. 2016). This was particularly the case for summer and autumn dry matter yield, while spring dry matter yield nearly remained unchanged (Sampoux et al. 2011). Traits such as heading and aftermath heading are important criteria for sward management, as with the beginning of the inflorescent period, the sward changes from vegetative to generative growth and is then dominated by reproductive tillers, generally resulting in a reduced biomass growth and forage quality (Hurley et al. 2007; McGrath et al. 2010). Plant breeders developed forage type ryegrass cultivars with different maturity groups, from early heading types with high early season yields to late heading types which are best adapted to a long grazing season and are used for permanent pastures due to their increased summer and autumn growth (Laidlaw 2005; Humphreys et al. 2010). Heading after cut is unwanted in forage production because a high share of reproductive tillers reduces the pasture quality in summer. A steady, but significant decrease in aftermath heading has thus been achieved for perennial ryegrass cultivars registered in national lists during the past decades (Sampoux et al. 2011). The resistance to diseases, especially to crown rust (*Puccina coronata* f. sp. *lolii*) as the most severe infection in ryegrass, improved in registered ryegrass cultivars for forage as well as for turf usage (Sampoux et al. 2011, 2013). On the other hand, modern ryegrass cultivars still suffer from adverse conditions in winter and frost in early spring (Hulke et al. 2007, 2008; Kemesyte et al. 2010). Furthermore, the level of tolerance of perennial ryegrass cultivars to heat and drought stress in early spring and summer is insufficient and the resistance to diseases, particularly nematodes, bacterial wilt and viruses needs to be improved (Easton et al. 2011; Hatier et al. 2014; Sanna et al. 2014). Finally, an increased nutrient and water uptake efficiency by studying the architecture and function of the roots gains more and more importance in forage and in turf grass breeding (Crush et al. 2010).

Wild and naturalized *L. perenne* populations (ecotypes) exhibit a high variability regarding those traits of interest, as they are adapted to the specific climatic conditions of their habitat. The collection and the thorough evaluation of perennial ryegrass ecotypes from diverse habitats enables breeders and researchers to identify suitable germplasm for breeding programs and to broaden the genepool available for ryegrass breeding. Several collections of *L. perenne* ecotypes and subsequent evaluations have thus been carried out since the 1980s. Charmet and colleagues evaluated natural perennial ryegrass populations collected in France, Germany, Ireland, Norway, Romania and Wales and identified populations which performed better than standard varieties in terms of rust resistance, spring growth, aftermath heading and winter hardiness (Charmet et al. 1989, 1990; Balfourier and Charmet 1991). In particular, Welsh populations combined high rust resistance with good growth scores, while accessions from Romania seemed to be promising germplasm to improve spring growth of perennial ryegrass cultivars (Charmet and Balfourier 1991). Germplasm with low rust susceptibility, high early spring growth and high autumn regrowth but strong aftermath heading was identified amongst *L. perenne* ecotypes collected in Galicia/Spain (Oliveira-Prendes and Charmet 1989). Later on, accessions from North West Spain made an important contribution to ryegrass breeding in New Zealand (Stewart 2006; Easton et al. 2011). Ecotypic germplasm from Hungary, Romania and Ukraine was found to be useful to improve the winter hardiness of perennial ryegrass cultivars (Humphreys 1989; Hulke et al. 2007, 2008; Kemesyte et al. 2010). Promising accessions with good turf quality and adaption to spring and summer drought were identified in perennial grass populations collected in Italy and Sardinia (Romani et al. 2002; Sanna et al. 2014).

The collection, maintenance, characterization and evaluation of the natural diversity of cultivated crops and their wild relatives is a key task of genebanks. As one of the largest genebanks in the world, the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) preserves and studies the genetic diversity of wild and cultivated plant genetic resources of about 151,000 accessions from 3000 botanical species. The Satellite Collections of the IPK Genebank at Malchow/Poel hold a comprehensive grass collection with more than 10,000 accessions. Amongst them, 3083 *L. perenne* accessions are currently under conservation, which have partly been evaluated in multi-year field trials for important agronomic traits. This paper presents results from the evaluation of 336 *L. perenne* genebank accessions originating from Bulgaria, Croatia, Spain and Ireland and aims to explore the diversity amongst this set of perennial ryegrass accessions in regard to plant productivity, winter hardiness and disease susceptibility, and to identify promising accessions for further research and plant breeding. Our study completes phenological information about natural ryegrass accessions originating from Spain and Ireland, and provides novel information on the agronomical performance of perennial ryegrass ecotypes from Croatia and Bulgaria.

Materials and methods

Collection of the *Lolium perenne* ecotypes

L. perenne ecotypes were collected in Bulgaria (BGR) in 1998, in Croatia (HRV) in 1996 and 1997, in Spain (ESP) in 1999 and in Ireland (IRL) in 2002. 45 sites were visited in Croatia, 60 in Bulgaria, 78 in Spain and 153 in Ireland, yielding altogether 336 ecotypic populations (accessions; Table 1). For each ecotype seeds from 30 spikes of different individual plants or 15–20 whole plants (including roots) were collected across an area of at least 5000 m^2. The collections focused on the north-west coast of Croatia and central Croatia, covered whole Bulgaria and Ireland (except Northern Ireland), and concentrated on the north western part of Spain (Galicia, Fig. 1). The ecotypes were collected from cultivated habitats such as fallow land, orchards, parks and old pastures where no ploughing or sowing took place in the previous 10 years (pers. comm. by the respective land owners). Wild habitats such as natural grasslands, woodlands or aquatic habitats as well as ruderal habitats like roadsites or field margins were also considered. The majority of the Croatian ecotypes was collected from cultivated and ruderal habitats, while a high proportion of the Bulgarian ecotypes was collected from wild habitats. Ecotypes from Spain and Ireland were predominately collected from old pastures and to a lesser extent from ruderal and wild habitats (Table 1). The collection sites ranged from 0 to 1360 m above sea level (asl), with sites above 1000 m asl only visited in Bulgaria. Based on the geographical coordinates of the sampling sites, the collection areas encompassed between 14,000 km^2 (Croatia) and 62,000 km^2 in Bulgaria. The climatic conditions at the sampling sites were obtained from the Global Climate Database (WorldClim) and are presented in Table 1. The sampling sites in Bulgaria represent a broad range of different climatic conditions with average annual temperatures from 2.8 to 13.3 °C and an annual precipitation from 514 to 740 mm, while the climatic conditions amongst the Spanish sampling sites were rather homogenous. After collection, the populations were transferred to the IPK Genebank, Satellite Collections North at Malchow/Poel, and were propagated during the years 1999–2003 in order to produce enough seeds for the subsequent evaluation trials. The passport data of the accessions and the data generated during the current study are available upon request or via the IPK Genebank Information System GBIS (https://gbis.ipk-gatersleben.de) and the European Search Catalogue for Plant Genetic Resources (https://eurisco.ipk-gatersleben.de).

Evaluation trials

Between 2002 and 2010, the 336 *L. perenne* accessions were evaluated regarding important agronomic traits at the Malchow/Poel station of the IPK in northern Germany (Longitude 11°28′26″E, Latitude 53°59′40″N, 10 m asl). The site is characterized by a mean annual precipitation of 591 mm and a mean annual air temperature of 8.9 °C (Table 2). The predominant soil type is sandy loam. Four single field trials were conducted. In each trial the ecotypes from the respective country were cultivated over a three year period (Table 1). The accessions were sown in plastic pots in a glasshouse and 4 × 10 plants of the respective accession were transplanted to the field in April. All the field trials were arranged in a completely randomized block design with four replicates, except the trial with the Bulgarian ecotypes where five replicates (blocks) were established. In each block 10 plants per accession were planted in a row, with a distance of 50 cm between the rows and a space of 10 cm between plants within one row (25 cm in the first block for single plant evaluations). In each of the four trials, eight commercially available cultivars were grown as a control (check varieties): Aberelf, Barclay, Cancan, Fennema, Gladio, Juwel, Respect, Sambin. The check varieties Barclay and Juwel are dedicated turf species, both characterized by an early heading and medium winter hardiness. Fennema, Sambin, Gladio, Respect, Cancan and Aberelf are forage types and characterized by medium to late heading, medium biomass production in the year of sowing, medium to high biomass production in the

Table 1 Collection year, total number of the *L. perenne* populations collected, collection sites as well as duration of the four separate field trials at the IPK research station Malchow/Poel, Germany

	Croatia	Bulgaria	Spain	Ireland
Collection year	1996/97	1998	1999	2002
No. of populations	45	60	78	153
Collection sites[a]				
Cultivated habitat	29 (64%)	22*(37%)	49 (63%)	110 (72%)
Ruderal habitat	12 (27%)	11 (18%)	13 (17%)	7 (5%)
Wild habitat	3 (7%)	16 (27%)	16 (20%)	31 (20%)
Other	1 (2%)	1 (2%)	–	5 (3%)
Geocoordinates of the collection sites				
Longitude (°, min/max)	14.6000/18.9333	23.0042/28.4008	− 9.2703/− 7.0756	− 10.2653/− 6.2500
Latitude (°, min/max)	44.7167/46.1167	41.5911/43.7686	42.2475/43.6831	51.4560/54.5187
Elevation (m, min/max)	0/800	0/1360	10/970	0/300
Area encompassed (km^2)	13,800	62,500	18,400	51,000
Climatic features of the collection sites[b]				
Mean annual temperature (°C)	9.9	10.5	12.4	9.3
Min/max (°C)	8.1/13.7	2.8/13.3	11/14.6	8.5/10.4
Mean annual precipitation (mm)	1120	593.4	1091	1033
Min/max (mm)	740/1356	514/740	913/1391	779/1461
Year of experiment	April 2003–April 2005	April 2002–April 2004	April 2004–May 2006	April 2008–April 2010

*Habitat information of ten populations is missing

[a]Coding scheme according to EURISCO standards, [b]Climatic data of the collection sites from WorldClim—Global Climate Database http://www.worldclim.org, data given as average of all the sampling sites, given is the average of the mean annual temperature from all the collection sites of the respective country as well as lowest and highest mean annual temperature among the collection sites, data for precipitation presented accordingly

first full harvest year and medium susceptibility for rust infection. In the following, the year of sowing/transplanting is named as the first experimental year, the year hereafter, which could be considered as the first full harvest year, is named as the second experimental year. The trial ended in the third experimental year after the final evaluation of the plant performance at the end of April. The traits investigated were observed on a plot level and scored on a visual scale ranging from 1 to 9.

1. The number of plants flowering in the first year (FLW1) was evaluated in July and was rated on a scale from 1 = one plant having inflorescences to 9 = nine to ten plants having inflorescences (early flowering).
2. Plant growth before winter was evaluated in the first (GBW1) and the second experimental year (GBW2) at the end of the vegetation period in late October or beginning of November and scorings ranged from: 1 = very weak to 9 = very good.
3. Growth in spring documented the plant growth at the end of April at 20 cm canopy height in the second (GIS2) and third (GIS3) experimental year and was assessed with: 1 = very low to 9 = very high.
4. Aftermath heading was evaluated in the second experimental year (AH2) approximately six weeks after the first cut and was rated from 1 = one plant having inflorescences to 9 = nine to ten plants having inflorescences.
5. Aboveground plant biomass (PBM2) reflects the biomass of the second cut in the second experimental year which was estimated at the beginning of July on a scale from 1 = very low to 9 = very high.

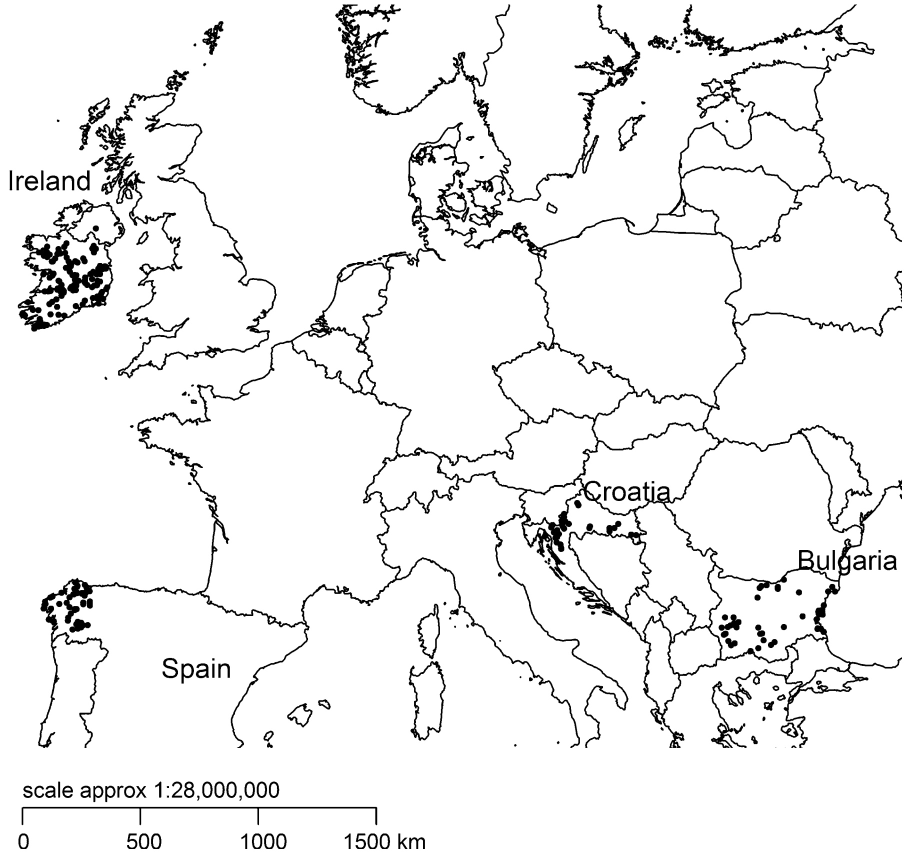

Fig. 1 Geographic distribution of the collection sites in Bulgaria, Croatia, Ireland and Spain

6. Rust (RUST2) and general disease (DIS2) susceptibility were evaluated during the period of maximal infection, generally in August or September in the second experimental year, and were rated on a scale from 1 = no symptoms to 9 = very susceptible. The evaluation of rust susceptibility considered predominately crown rust, while the evaluation of general disease susceptibility comprised a mixed infection with viruses, leaf spot and rust.
7. The plant growth after winter was assessed in the third experimental year (GAW3) at the beginning of the vegetation period in March with 1 = very weak to 9 = very good.

The maintenance of the trials followed common agricultural practice. As fertilizer, nitrogen (N) was applied annually in the form of calcium ammonium nitrate (CAN) amounting to 80 kg N per hectare in early spring and 60 kg N per hectare after each cut. Three cuttings were realized during the first full harvest year (second experimental year). The climatic conditions during the experimental years are given in Table 2. The mean monthly air temperature during the experimental period was slightly higher compared to the long-term average, in particular in the years 2008 and 2009 (Irish ecotypes, first and second experimental year), with mild winters and a hot and dry spring or summer. The average annual rainfall was exceptionally high in 2002, the first experimental year of the Bulgarian ecotypes, with heavy rainfall from July to October, and low rainfall in the year 2008, with a particularly dry period from May to July 2008.

Statistical analyses

The statistical analyses were performed using the software package R (Version 3.3.2). The four trials were conducted in different years and thus under varying climatic conditions. To allow for a rough comparison of the *L. perenne* accessions of the different trials, the scores of each accession in the particular trial were subtracted by its corresponding

Table 2 Mean monthly air temperature (°C) and monthly precipitation (mm) during the four different phenotyping experiments at the IPK research station Malchow/Poel, Germany, as well as their long-term average

Year	2002	2003	2004	2005	2006	2008	2009	2010	1977–2010
Average air temperature (°C)									
January	2.3	0.5	− 1.0	3.8	− 1.5	4.5	0.5	− 3.7	0.8
February	4.7	− 1.5	2.9	0.8	1.3	5.3	1.4	− 0.6	1.0
March	3.9	4.2	4.4	3.3	1.3	5.2	5.5	5.0	3.9
April	7.3	8.6	9.4	8.5	8.2	8.6	11.6	8.8	7.5
May	14.5	13.3	12.0	12.8	12.8	14.2	13.5	10.5	12.6
June	17.6	17.9	14.7	15.8	16.7	17.5	15.1	16.0	15.4
July	18.3	19.7	16.7	18.0	22.5	19.4	19.4	22.0	17.7
August	20.4	19.3	19.1	16.4	17.7	18.5	19.9	18.3	17.2
September	15.9	15.5	14.2	16.0	17.5	14.1	16.2	14.2	13.8
October	8.4	6.7	10.3	10.8	13.3	10.6	8.9	9.8	9.7
November	4.0	6.6	5.6	4.7	8.2	6.9	8.0	5.3	4.9
December	− 0.6	3.1	3.7	1.2	6.6	3.0	1.1	− 3.2	1.9
Average	9.7	9.5	9.3	9.3	10.4	10.7	10.1	8.5	8.9
Precipitation (mm)									
January	34.5	38.3	39.8	50.5	14.7	46.9	14.8	39.2	40.9
February	82.8	8.0	60.4	41.5	36.5	13.7	51.2	32.4	35.0
March	29.5	17.4	25.2	29.2	55.4	50.2	47.0	61.9	41.4
April	55.2	38.0	37.0	23.9	53.0	31.6	4.4	22.4	34.4
May	60.7	60.2	55.8	59.8	53.2	9.4	34.3	75.8	45.8
June	72.4	51.1	81.3	35.6	34.6	12.0	60.1	42.4	61.1
July	125	92.6	87.4	155	22.5	25.5	114	30.8	67.5
August	123	47.6	126	29.8	127	105	32.8	126	69.5
September	16.3	43.6	64.0	40.7	46.7	27.6	42.4	114	53.3
October	100	48.5	34.0	46.0	33.6	57.0	80.8	55.8	45.6
November	91.7	29.6	43.6	34.5	67.8	26.3	100	113.3	50.8
December	22.3	42.4	24.0	75.3	23.0	56.7	44.2	31.0	46.7
Sum	813	517	679	623	568	462	626	745	592

"year effect" as described in Bothe et al. (2016) previously.

To compute the year effect, the following equation was used:

$$C_i = \overline{X_i} - \sum_{i=1}^{n} \frac{\overline{x_i}}{n}$$

where C_i is the effect of the calendar year, $\overline{X_i}$ is the mean of the check varieties in the *i*th year and n is the number of trials. Due to the adjustment for the "year effect", scores below 1 and above 9 also occurred in a few cases. In a first step, each trial was analysed separately. Mean as well as minimum (min) and maximum (max) values were calculated for each trait. Additionally, multiple comparisons to the control (check mean) were computed. Therefore, a two-way analysis of variance was conducted based on a general linear mixed model were the population was considered as fixed effect and the repetition (block) was considered as random effect using the "lmer" procedure of the R package "lme4" (Bates et al. 2015). If a significant effect of the factor population was determined, post hoc comparisons to the control (check mean) were computed using the Dunnett's test at a significance level of $\alpha \leq 0.05$ (package "multcomp", Hothorn et al. 2008). If the normality of the residuals and the homogeneity of variances was not given, the Kruskal–Wallis-test and the respective Dunn's test against one control of the package PMCMR was applied (Pholert 2014). The latter was the case for the trait FLW1 in all the trials, the RUST2 and DIS2 traits in the trials comprising the Croatian, Bulgarian and

Irish ecotypes, but the traits GBW1, PBM2, GAW3 in the trial with the Spanish ecotypes. Based on the ANOVA results and the post hoc test, the percentage of populations achieving scores significantly higher or lower than the control was calculated. In a second step, all the trials were analyzed together for the relationship between the ten traits investigated using the Spearman correlation coefficient (except the check varieties). The structure of the dataset was further studied by applying a principal component analysis and using the "PCA" procedure of the package "FactoMineR" (Le et al. 2008). This analysis was based on the means of all the 336 *L. perenne* populations, and on all the traits evaluated. Additionally, the means of the check varieties, grouped into turf type cultivars and forage type cultivars, were included in this analysis. For a more detailed description of the collections sites, climatic variables such as annual mean temperature, annual precipitation and minimum temperatures of the coldest month were downloaded from the World Climate Database (www.worldclim.org) using the R "raster" package (Hijmans 2016).

Results

Performance of the ecotypes in the respective field trial

Ecotypes collected in Bulgaria

In general, the ecotypes collected in Bulgaria showed a high variability regarding most of the traits investigated and clearly differed from the check varieties. The majority of the ecotypes did not flower in the first experimental year (mean 1.2, Table 3). The plant growth before winter in the first year was on average scored with 6.5 for the ecotypes, and was generally lower compared to that of the check varieties. However, individual scorings varied between 5.3 and 8.1, reflecting a broad spectrum of medium to very well developed populations. In comparison to the check mean, 63% of the populations had significantly lower mean scores, and 37% did as well as the check varieties. In contrast, the ecotypes grew well in spring of the second year (mean 7.9). About 58% of the accessions obtained significantly higher scores than the check mean, and six accessions even surpassed the scorings of the best cultivar: GR6562, GR6563, GR6586, GR6592, GR6601, GR6609. Aftermath heading was scored on average with 2.9 and was comparable to that of the respective check varieties, indicating a low development of reproductive tillers after the first cut. The plant biomass development at the second cut was low for the ecotypes (mean 3.7), and 88% of them had significantly lower yield scores compared to the check mean. However, a high ecotypic variation was observed for this trait with scorings ranging between 1.5 and 6.3. The accessions which achieved highest yields, comparable to that of the check mean, were: GR6557, GR6560, GR6562, GR6563, GR6601, GR6603, GR6609. Almost no rust symptoms were observed for the ecotypes as indicated by scorings between 1.4 and 2.2, while the respective check varieties were more sensitive to rust infection (mean 3.6, Table 3). The susceptibility to general diseases such as virus and leaf spot was higher than for rust and varied between 2.2 and 5.6 for the ecotypes, which in most of the cases did not differ from the check mean. Regarding plant growth before winter in the second year, the ecotypes again differed strongly. Scorings ranged between 3.2 and 8.0, and about 43% of the Bulgarian ecotypes grew significantly worse than the check varieties and achieved even lower scorings than the ecotypes from Spain, Croatia and Ireland. On the other hand, about 52% of the Bulgarian accessions grew as well as the checks and 5% even surpassed the average performance of the checks. The best accessions, with scorings above 7.8 were: GR6562, GR6563, GR6601. The same trend was observed in the beginning of the third experimental year. On average, growth after winter of the ecotypes was weak (mean 3.3). Compared to the check mean, 85% of the Bulgarian ecotypes had significantly lower mean scores and ranged even behind the ecotypes from Croatia, Spain and Ireland. Interestingly, until late spring, the growth score of the Bulgarian accessions increased to 5.5, and more than 70% of the accessions were comparable to check mean, while four accessions even surpassed the average performance of the check varieties: GR6557, GR6562, GR6563, GR6609 (Table 3).

Ecotypes collected in Croatia

The ecotypes collected in Croatia generally equaled the performance of the check varieties regarding most of the traits investigated. For the majority of the

Table 3 Mean as well as minimum and maximum values and results of analysis of variance for ten visually scored traits of the ecotypes collected in Bulgaria (BGR) and eight commercially available *L. perenne* varieties cultivated in a three year field trial under maritime conditions

Year	Trait	Ecotypes (BGR)*		Check varieties		ANOVA	Post hoc test against control (check mean)		
		Mean	Range	Mean	Range	Population (p value)	+ % of populations	=	−
1	FLW	1.2	1.1–2.5	1.1	1.1–1.1	< 0.001^	3.30	**96.7**	0.00
1	GBW	6.5	5.3–8.1	7.8	7.3–8.3	< 0.001	0.00	36.7	**63.3**
2	GIS	7.9	5.6–9.6	7.0	4.4–8.2	< 0.001	**58.3**	40.0	1.70
2	AH	2.9	1.8–5.1	2.9	2.3–3.5	< 0.001	10.0	**90.0**	0.00
2	PBM	3.7	1.5–6.3	5.8	3.7–7.5	< 0.001	0.00	11.7	**88.3**
2	RUST	1.5	1.4–2.2	3.6	2.6–5.2	< 0.001^	0.00	0.00	**100**
2	DIS	4.0	2.2–5.6	3.3	2.6–4.0	< 0.001^	1.70	**98.3**	0.00
2	GBW	6.0	3.2–8.0	6.7	6.0–7.8	< 0.001	5.00	**51.7**	**43.3**
3	GAW	3.3	2.4–4.1	4.4	2.7–5.3	< 0.001	0.00	15.0	**85.0**
3	GIS	5.5	4.3–7.3	5.9	3.3–6.7	< 0.001	6.67	**75.0**	18.3

FLW flowering tendency, *GBW* growth before winter, *GAW* growth after winter, *GIS* growth in spring, *AH* aftermath heading, *PBM* biomass, *RUST* rust susceptibility, *DIS* disease susceptibility

*Scoring between 2002 and 2004 of 60 ecotypic populations collected in Bulgaria and 8 check varieties, ^Non-parametrical ANOVA (Kruskal–Wallis-test), +: significantly higher, =: equal, −: significantly lower

Table 4 Performance of the *L. perenne* ecotypes collected in Croatia (HRV) cultivated in three year field trials under maritime conditions. Mean as well as minimum and maximum values and results of analysis of variance for ten visually scored traits

Year	Trait	Ecotypes (HRV)*		Check varieties		ANOVA	Post hoc test against control (check mean)		
		Mean	Range	Mean	Range	Population (p value)	+ % of populations	=	−
1	FLW	1.3	1.1–5.9	1.1	1.1–1.1	< 0.001^	4.40	**95.6**	0.00
1	GBW	7.8	5.3–9.1	7.8	5.9–9.6	< 0.001	13.3	**77.8**	8.90
2	GIS	6.8	5.1–8.8	7.0	5.8–8.1	< 0.001	2.22	**84.4**	13.3
2	AH	3.5	2.4–5.1	2.9	2.4–3.6	< 0.001^	0.00	**100**	0.00
2	PBM	5.6	5.1–6.3	5.8	5.1–7.1	0.117	0.00	**100**	0.00
2	RUST	3.4	2.7–4.0	3.6	3.2–4.2	0.039	6.67	**93.3**	0.00
2	DIS	3.4	2.9–4.2	3.3	3.2–3.4	0.618^	0.00	**100**	0.00
2	GBW	6.6	5.6–7.1	6.7	5.6–7.4	0.038	0.00	**97.8**	2.22
3	GAW	4.5	3.5–5.3	4.4	3.6–4.8	< 0.001	11.1	**88.9**	0.00
3	GIS	5.6	4.7–6.7	5.9	3.7–6.7	0.034	0.00	**97.8**	2.22

FLW flowering tendency, *GBW* growth before winter, *GAW* growth after winter, *GIS* growth in spring, *AH* aftermath heading, *PBM* biomass, *RUST* rust susceptibility, *DIS* disease susceptibility

*Scoring between 2003 and 2005 of 45 ecotypic populations collected in Croatia and 8 check varieties, ^Non-parametrical ANOVA (Kruskal–Wallis-test), +: significantly higher, =: equal, −: significantly lower

accessions, no flowering was observed in the year of sowing (average score 1.3, Table 4). Only two accessions had higher scores, causing the high variation observed for this trait: GR 6309 (score 2.1) and GR 6379 (score 5.9). The ecotypes grew well before the first winter, and, in contrast to the Bulgarian ecotypes, exhibited a performance similar to or better than that of the check varieties. The best accessions in regard to this trait were: GR6337, GR6383, GR6384, GR6385, GR6386 and GR6392. Additionally, with an average score of 6.8 and minimum and maximum values of 5.1 and 8.8, the ecotypes were medium to well developed in spring in the second experimental year. In comparison to the check mean, about 84% of the accessions equaled the average spring growth of the standard varieties. Although a statistical significant effect of the factor population was indicated by the non-parametrical ANOVA, no significant difference between the ecotypes and the respective checks was detected in terms of aftermath heading. Furthermore, the ecotypes equaled the check varieties regarding plant biomass development after cut. The incidence of disease symptoms was generally low in this trial (score ≤ 3.6) and the ecotypes did as well as the check varieties, irrespective whether rust or general pests, such as virus or leaf spot were considered. Similar to the check varieties, the ecotypes grew well before winter and were on a medium to weak level after winter. Compared to the check mean, five accessions showed a significantly better performance after winter (score > 5.0): GR5989, GR6307, GR6309, GR6345, GR6395. The quality of plant growth improved until late spring with scorings ranging between 3.7 and 6.7, and the majority of the ecotypes grew as well as the check varieties after three years of cultivation (Table 4).

Ecotypes collected in Galicia/Spain

The Spanish ecotypes exhibited a high productivity during the first two experimental years but showed a low variability and a uniform performance regarding most of the traits investigated, as shown by the respective ANOVA results (Table 5). No flowering was observed in the year of sowing (average score 1.3) and all the tested ecotypes were well developed before winter with scorings between 7.3 and 7.8. Furthermore, the majority of the ecotypes was well developed in spring of the second year, and achieved scorings comparable to or better than the check mean. Although not statistically significant, the Spanish ecotypes tended to a slightly stronger aftermath heading (mean 3.9) than the respective check varieties and the ecotypes from the other countries. As also observed for the ecotypes collected in Croatia, the plant biomass development of the Spanish ecotypes at the second cut was medium to high, and close to that of the check varieties. The susceptibility to diseases such as leaf spot, virus or rust was low and within the range of the respective check varieties and the ecotypes from the other countries. The ecotypes were well developed before winter in the second experimental year and exhibited a weak to medium growth after winter, similar to the respective check varieties. However, the plant growth in spring after three years of cultivation was highly variable amongst the ecotypes and partly remained on a low level. Compared to the respective check mean, about 42% of the Spanish accessions exhibited a significantly lower spring growth (scores ≤ 4.3) and stayed behind the average development of the ecotypes from the other countries (Tables 3, 4 and 5).

Ecotypes collected in Ireland

The majority of the Irish ecotypes exhibited a performance similar to that of the check varieties, but revealed interesting characteristics in terms of aftermath heading, disease susceptibility and plant growth after winter (Table 6). Flowering tendency in the first experimental year for the ecotypes was, on average, low. But unlike in the other trials, a broader range of scorings (0.6–3.8) was found for the trait flowering tendency, for the ecotypes as well as for the respective check varieties (Table 6). Similar to the Croatian and Spanish ecotypes, the majority of the Irish accessions (78%) developed well before winter, and did not significantly differ from the check mean. A stronger ecotypic differentiation was observed in terms of spring growth in the second experimental year. Scorings ranged between 3.6 and 7.6, indicating a diverse set of low to highly developed swards. About 80% of the Irish ecotypes did not differ from the check mean, but 19% of them had significantly lower scores (< 5.3), partly even lower than the minimum scores of the Bulgarian, Croatian and Spanish ecotypes. Interestingly, the Irish ecotypes exhibited a low aftermath heading. With an average score of 1.6, aftermath heading was clearly below that of the check varieties and that of the ecotypes from the other countries. In comparison to the check mean, 70% of the ecotypes exhibited a significantly lower aftermath heading. The average biomass development of the Irish accessions at the second cut was on a medium level, with a high

Table 5 Results of the three year field trial with *L. perenne* ecotypes collected in Spain (ESP) and the respective check varieties. Mean, minimum and maximum values as well as results of analysis of variance and post hoc comparison of means for the ten visually scored traits

Year	Trait	Ecotypes (ESP)*		Check varieties		ANOVA	Post hoc test against control (check mean)		
		Mean	Range	Mean	Range	Population (p value)	+ % of populations	=	−
1	FLW	1.3	1.1–2.1	1.1	1.1–1.1	< 0.001^	3.85	**96.1**	0.00
1	GBW	7.8	7.3–7.8	7.8	7.6–7.8	0.650^	0.00	**100**	0.00
2	GIS	7.5	5.7–8.4	7.0	4.9–8.2	< 0.001	35.9	**60.3**	3.84
2	AH	3.9	2.4–5.4	2.9	2.2–4.2	0.853^	0.00	**100**	0.00
2	PBM	5.9	3.0–6.7	5.8	4.0–6.5	0.108^	0.00	**100**	0.00
2	RUST	3.8	3.2–4.5	3.6	3.5–3.7	0.623	0.00	**100**	0.00
2	DIS	3.5	2.8–4.1	3.3	3.1–3.6	0.714	0.00	**100**	0.00
2	GBW	6.5	5.2–8.2	6.7	6.0–7.5	< 0.001	1.28	**94.9**	3.85
3	GAW	4.0	3.3–4.6	4.4	4.1–4.8	< 0.001^	0.00	**100**	0.00
3	GIS	4.5	2.5–6.5	5.9	5.3–6.3	< 0.001	0.00	**57.7**	**42.3**

FLW flowering tendency, *GBW* growth before winter, *GAW* growth after winter, *GIS* growth in spring, *AH* aftermath heading, *PBM* biomass, *RUST* rust susceptibility, *DIS* disease susceptibility

*Scoring between 2004 and 2006 of 78 ecotypic populations collected in Spain and 8 check varieties, ^Non-parametrical ANOVA (Kruskal–Wallis-test), +: significantly higher, =: equal, −: significantly lower

Table 6 Mean, minimum and maximum values for the *L. perenne* ecotypes collected in Ireland (IRL) and the respective check varieties as well as results of analysis of variance and post hoc comparison of means for the ten visually scored traits in the three year field trial

Year	Trait	Ecotypes (IRL)*		Check varieties		ANOVA	Post hoc test against control (check mean)		
		Mean	Range	Mean	Range	Population (p value)	+ % of populations	=	−
1	FLW	1.1	0.6–3.8	1.1	0.6–1.9	< 0.001^	0.00	**100**	0.00
1	GBW	7.2	5.8–8.0	7.8	7.0–8.3	< 0.001	0.00	**77.8**	22.2
2	GIS	6.2	3.6–7.6	7.0	5.1–8.1	< 0.001	0.00	**81.0**	19.0
2	AH	1.6	0.5–3.0	2.9	2.3–3.3	< 0.001	0.00	29.4	**70.6**
2	PBM	5.0	3.5–6.2	5.8	3.7–7.7	< 0.001	0.00	**62.0**	**37.9**
2	RUST	3.3	1.9–4.9	3.6	3.1–3.9	0.192^	0.00	**100**	0.00
2	DIS	2.9	2.1–4.9	3.3	2.9–3.6	< 0.001	0.65	**89.5**	9.80
2	GBW	6.7	5.1–7.8	6.7	5.6–7.3	< 0.001	0.65	**96.1**	3.27
3	GAW	5.4	3.2–7.2	4.4	2.7–5.5	< 0.001	20.2	**79.7**	0.00
3	GIS	6.0	3.4–7.9	5.9	3.2–7.0	< 0.001	3.27	**88.9**	7.84

FLW flowering tendency, *GBW* growth before winter, *GAW* growth after winter, *GIS* growth in spring, *AH* aftermath heading, *PBM* biomass, *RUST* rust susceptibility, *DIS* disease susceptibility

*Scoring between 2006 and 2008 of 153 ecotypic populations collected in Ireland and 8 check varieties, ^Non-parametrical ANOVA (Kruskal–Wallis-test), +: significantly higher, =: equal, −: significantly lower

percentage of the accessions (38%) achieving significantly lower scores than the check mean. As already observed in the other trials, the incidence of rust and general disease symptoms was moderate and did not exceed a maximal scoring of 4.9. The majority of the accessions did not differ from the check mean, but minimum values of 1.9 (RUST2) and 2.1 (DIS2) were also recorded, which were clearly below the minimum values of the check varieties. A particular low susceptibility to general diseases accompanied by a low susceptibility to rust was observed for the accessions GR7600, GR7707 and GR7724. Similar to the Croatian and Spanish ecotypes, the Irish entries grew well before winter in the second experimental year, but were remarkably well developed at the beginning of the vegetation period of the third experimental year, with maximum values at 7.2—a level which was neither achieved by the respective check varieties nor by the ecotypes from the other countries. Compared to the check mean, about 20% of the ecotypes grew significantly better after winter. The highest scores ($\geq$ 7.0) were obtained by the accessions: GR7607, GR7612, GR7622, GR12044, GR12053, GR12302, GR12306, GR12318. Based on the good development after winter, the Irish accessions achieved high scorings for spring growth in the third experimental year, but, as already observed in the second experimental year, a strong differentiation amongst the ecotypes was observed with scores ranging between 3.4 and 7.9. While the majority of the Irish accessions (89%) did not differ significantly from the check mean, five accessions were significantly better (GR7607, GR7793, GR12038, GR12044, GR12053) and twelve accessions were less developed.

Correlation amongst the investigated traits and results of the principal component analysis

To investigate the relationships between the scored traits, the results of the 336 *L. perenne* accessions were subjected to Spearman rank correlation analysis and principal component analysis (PCA). The correlation amongst the traits was generally medium to weak ($r_s < 0.50$). However, a strong positive monotonic correlation was observed between growth before winter in the first scoring year (GBW1) and biomass production in the second scoring year (PBM2, Table 7). Thus, the better the growth in the first year, the better the yield of the ecotypes was at the second cut in the following year. Furthermore, there was a moderately positive correlation between the trait growth in spring in the second scoring year (GIS2) and aftermath heading (AH2). A moderately negative correlation occurred between aftermath heading (AH2) and growth after winter in the third scoring year (GAW3). This indicates that ecotypes with a strong growth in spring tended to a faster aftermath heading and, at the same time, to a reduced growth after winter in the third year. Furthermore, a strong positive correlation was observed between the trait growth after winter (GAW3) and the trait growth in spring (GIS3) in the third experimental year, showing that a fast and good growth after winter led to a good development later in spring. Unexpectedly, this data set revealed a moderate positive correlation between susceptibility to rust infection (RUST2) and plant biomass development (PBM2) later in the year. This indicated that the higher susceptibility to rust infection was, the better developed were the plants.

The traits included in the PCA were the adjusted accession means and all the evaluated parameters. As shown in Table 8, the first three components explained together 68% of the total variation in the data set. The first component (Dim1) explained 31%, while the second component (Dim2) explained 24% and the third component (Dim3) explained 14% of the total variation. The most important traits contributing to the first component were plant biomass (PBM2), rust (RUST2) and disease susceptibility (DIS2) as well as growth before winter (GBW2) in the second experimental year, and growth after winter (GAW3) in the third experimental year. The second component was mainly explained by growth before winter in the year of sowing (GBW1), growth in spring (GIS2) and aftermath heading (AH2) in the second year. The trait flowering tendency (FLW1) did not contribute much to the principal components, and, hence, to explain the differentiation in the data set.

As can be seen from the projection of the accessions on the biplot of the first two dimensions, the different *L. perenne* accessions grouped together according to their provenances (Fig. 2). The Spanish and the Croatian clusters overlapped and were located close to the cluster of the check varieties, particularly the forage type cultivars. While the Croatian accessions showed a stronger scattering on the plot, the Spanish accessions densely grouped together, underpinning the low ecotypic variation in this data set. The accessions collected in Spain and Croatia were preliminary associated with a higher aftermath heading as well as a favourable growth in spring in the second year (GIS2), growth before winter in the first year (GBW1) and plant biomass development in the

Table 7 Correlation matrix of the ten agronomic traits evaluated during the three year field trials with 336 *L. perenne* accessions collected in Bulgaria, Croatia, Spain and Ireland (Spearman correlation coefficient r_s)

	FLW1	GBW1	GIS2	AH2	PBM2	RUST2	DIS2	GBW2	GAW3	GIS3
FLW1	1									
GBW1	0.20 **	1								
GIS2	0.37 ***	0.34 ***	1							
AH2	0.46 ***	0.44 ***	**0.54 *** **	1						
PBM2	0.16 n.s.	**0.70 *** **	0.22 ***	0.38 ***	1					
RUST2	0.02 n.s.	0.49 ***	− 0.08 n.s.	0.18 *	**0.59 *** **	1				
DIS2	0.23 ***	0.05 n.s.	0.44 ***	0.49 ***	0.004 n.s.	0.11 n.s.	1			
GBW2	0.11 n.s.	0.33 ***	− 0.03 n.s.	− 0.09 n.s.	0.26 ***	0.14 n.s.	− 0.22 ***	1		
GAW3	− 0.30 ***	0.01 n.s.	− 0.49 ***	**− 0.51 *** **	0.11 n.s.	0.20 **	− 0.34 ***	0.38 ***	1	
GIS3	− 0.14 n.s.	− 0.04 n.s.	− 0.12 n.s.	− 0.33 ***	0.03 n.s.	− 0.09 n.s.	− 0.22 **	0.43 ***	**0.70 *** **	1

FLW flowering tendency, *GBW* growth before winter, *GAW* growth after winter, *GIS* growth in spring, *AH* aftermath heading, *PBM* biomass, *RUST* rust susceptibility, *DIS* disease susceptibility

***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$; *n.s.* not significant

Table 8 Eigenvalues and percent of variance explained by the first three principal components as well as the correlation of the investigated traits to the principal components

	PC1	PC2	PC3
Eigenvalue	3.05	2.32	1.36
% cum. variance	30.5	54.4	68.4
Correlation of traits with the principle components			
FLW1	− 0.03	0.31	0.34
GBW1	0.61	**0.58**	0.17
GIS2	− 0.22	**0.67**	0.55
AH2	− 0.22	**0.84**	− 0.01
PBM2	**0.72**	0.53	− 0.13
RUST2	**0.69**	0.28	− 0.46
DIS2	**− 0.62**	0.32	0.21
GBW2	**0.70**	0.12	0.34
GAW3	**0.73**	− 0.44	0.21
GIS3	0.42	− 0.34	**0.74**

FLW flowering tendency, *GBW* growth before winter, *GAW* growth after winter, *GIS* growth in spring, *AH* aftermath heading, *PBM* biomass, *RUST* rust susceptibility, *DIS* disease susceptibility

second year (PBM2), and their performance was closely related to the performance of the forage type check varieties. The ecotypes collected in Bulgaria formed a separate cluster which was predominately located at the negative direction of both dimensions, demonstrating that the Bulgarian ecotypes were mainly associated with a lower growth before winter (GBW1, GBW2), plant biomass development at the second cut (PBM2), low susceptibility to rust infection, but a slightly higher susceptibility to general diseases. The Bulgarian cluster strongly scattered on the biplot, reflecting the high variation in this data set. This is underpinned by three accessions of the Bulgarian ecotypes (GR6562, GR6563, GR6601) which can be found amongst the cluster of the forage type check varieties and the Croatian ecotypes on the biplot. The Irish accessions clustered together, were predominately located on the negative direction of dimension 2, and were closely related to a good development after winter (GAW3), a higher spring growth in the third experimental year (GIS3) and a low aftermath heading in the second year (AH2) (Fig. 2).

Discussion

The performance of perennial ryegrass ecotypes collected in Spain, Bulgaria, Croatia and Ireland was evaluated in three year field trials under the mild maritime climatic conditions of the Island of Poel (Germany). A fundamental criterion in ryegrass characterization and evaluation is its reproductive development, comprising traits such as heading, ear emergence and flowering. This trait, which even helps

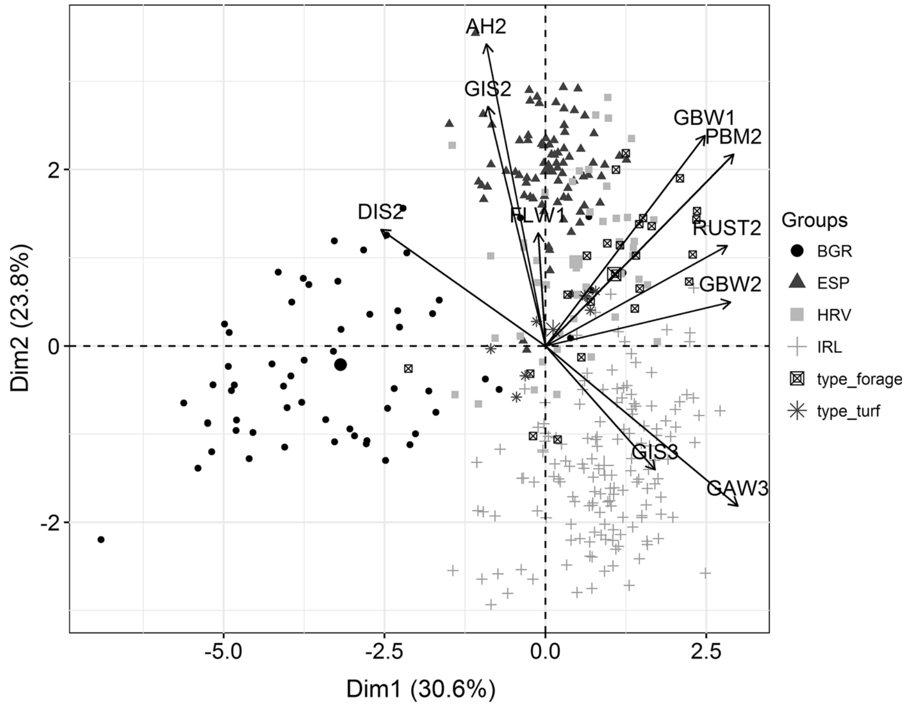

Fig. 2 Biplot of the principal component analysis of the first two dimensions for the 336 *L. perenne* accessions and the respective forage and turf type check varieties and the traits evaluated (*FLW*, flowering tendency, *GBW* growth before winter, *GAW* growth after winter, *GIS* growth in spring, *AH* aftermath heading, *PBM* biomass, *RUST* rust susceptibility, *DIS* disease susceptibility)

to distinguish between several ryegrass species, determines sward quality and usability, particularly in forage productions (Hurley et al. 2007; Bennet et al. 2000; Wims et al. 2014). While annual ryegrass species such as Italian ryegrass (*L. multiflorum* Lam.), Annual ryegrass (*L. rigidum* Gaud.), or Darnel ryegrass (*L. temulentum* L.) do not need cold induction to head, most perennial ryegrass accessions will not head without low temperature exposure, and hence, stay vegetative in the year of sowing (Bennet et al. 2000; Humphreys et al. 2010). Considering all the ecotypes in the present study, the majority showed scorings for flowering tendency in the year of sowing lower than 2.1, what indicates that no flowering took place in the first year and underpins the perennial character of the collected ecotypes. A higher variation with regard to this trait was only observed in the trial with the Irish ecotypes. That might have been caused by the dry spring in the year 2008, where the monthly rainfall was only 9.4 mm in May and 12 mm in June, and thus clearly below the long-term average precipitation of the experimental site and the other trials (45.8 and 61.1 mm, respectively; Table 2). That drought stress may lead to a strong differentiation in flowering time has also been reported by Volaire and Lelièvre (2004) for *Dactylis glomerata* L. ecotypes. Flowering in the second year, in particular heading after cut, is unwanted, as with the beginning of the heading the number of reproductive tillers increases and the digestibility of the dry matter decreases (Hurley et al. 2007; McGrath et al. 2010;). Therefore, the low aftermath heading of the Irish ecotypes in the second experimental year, which was even below that of the ecotypes from Bulgaria, Spain and Croatia and that of the respective check varieties, is interesting for forage and turf grass breeding. Our results are in accordance with findings of Charmet et al. (1989) who studied 60 wild populations of *L. perenne* collected in Ireland under French conditions. They observed a lower aftermath heading of the Irish ecotypes compared to the commercial cultivars. As well, for some wild *L. perenne* populations collected all over France, a lower aftermath heading compared to that of the control varieties was reported (Charmet et al. 1990). In contrast, Oliveira-Prendes and Charmet (1989) described a stronger aftermath heading for *L. perenne* populations collected in North West Spain compared to the respective check varieties. The ability to flower in the sowing year and aftermath heading in *L. perenne* is strongly influenced by latitude, temperature and aridity of the collection site, and the warmer a climate becomes, for instance in a southern region, the more alternative and aftermath heading populations become (Balfourier and Charmet 1991). The place of origin might thus explain the variation in aftermath heading in our data set, with the low aftermath heading of the Irish ecotypes collected from northern latitudes and the slightly stronger aftermath heading of the ecotypes collected from southern latitudes like Croatia and Spain.

Growth activity and winter hardiness of *L. perenne* populations are important features for the productivity and persistency of ryegrass systems and were evaluated by the traits growth before and after winter, growing in spring and plant biomass development after cut. Especially in the first two experimental years, the Spanish ecotypes exhibited a strong growth activity, partly exceeding that of the check varieties and that of the ecotypes from Bulgaria and Ireland. Accordingly, Stewart (2006) and Oliveira-Prendes and Charmet (1989) described germplasm of *L. perenne* collected in North West Spain as having a good growth behavior during winter, a strong spring growth and autumn-regrowth. The strong growth activity of the Spanish ecotypes might be attributed to the high annual mean temperatures and high rainfall at the collection sites in Galicia, ranging between 11 and 14 °C and 900 and 1300 mm (Table 1). This causes good growing conditions throughout the year and results in actively growing populations even in the winter months. At the same time, this germplasm might be less adapted to adverse conditions in winter or early spring. Although the Spanish ecotypes grew well in the first and the second experimental year, growth in spring was low in the third experimental year under the respective field experimental conditions. These findings may be attributed to the unusual cold temperatures in spring 2006, with an average daily air temperature of 1.3 °C in March (long term average 3.9, Table 2) and minimal daily temperatures of − 9.0 °C (data not shown). Winter kill can occur due to extreme temperature fluctuations, a lacking snow cover, wind desiccation, diseases (snow mold) and drought during winter and in early spring, but freezing seems to be the most important component (Humphreys 1989; Hulke et al. 2008). The minimum lethal temperature (LT50) for perennial ryegrass accessions originating from Europe was determined to be between − 3.0 and − 13.9 °C (Humphreys and Eagles 1988; Hulke et al. 2008). Thereby, freezing tolerance strongly depends on the latitude and the climatic conditions during the winter month at the site of origin and a high freezing tolerance was reported for populations originating from continental or alpine climate i.e. from Norway, Sweden, Romania, Poland and Hungary (Humphreys and Eagles 1988; Hulke et al. 2007). The mild climate of Galicia with average temperatures of 7.3 °C and minimum temperatures rarely below 1 °C during the coldest month does not favor adaptation to frost events. The same was reported by Kreyling et al. (2012) who observed that spring frost tolerance was less developed in ecotypes of common grass species from sites with warmer spring temperatures than in ecotypes originating from areas with lower spring temperatures. In addition, actively growing, winter green populations could be even more damaged by late winter or early spring frosts (Humphreys 1989). Interestingly, the ecotypes collected in Ireland exhibited a good growth after winter in the third experimental year and partly exceeded the performance of the Bulgarian, Croatian and Spanish ecotypes, as well as the average performance of the respective standard varieties. The Irish ecotypes are not expected to be frost tolerant due to the mild winter climate at the site of origin, what has to be considered when cultivated under German conditions (Nehrlich et al. 2013). Correspondingly, Irish wild populations revealed a higher frost susceptibility than the commercial cultivars under French field conditions (Charmet et al. 1989). However, in this study the Irish accessions which were exposed to low temperatures in the winter 2009/10, with values below the long-term average in December (1.1 °C), January (− 3.7 °C) and February (− 0.6 °C, Table 2), and minimum temperatures in January and February down to − 15.3 °C (data not shown), performed rather well. This might be due to a long lasting snow-cover (pers. comm. Evelin Willner) that might have protected the populations during the severe frosts in January and February against freezing and wind desiccation.

The growth behavior of the Bulgarian ecotypes can be characterized by a low biomass production in summer after cut and before winter, but a strong biomass production in early spring. A low growth in autumn and during winter might be an advantageous strategy to withstand frost periods in the winter season and was also observed for *L. perenne* ecotypes collected in Hungaria and Poland (Humphreys 1989). Our study additionally showed that the Bulgarian ecotypes compensated the low autumn and winter growth until spring, since these showed a similar or better spring growth (GIS1, GIS2) than the ecotypes from Croatia, Spain and Ireland and the respective check varieties. Thus, Bulgarian ecotypes provide interesting germplasm to improve winter hardiness and spring growth in ryegrass cultivars. In contrast to the other collections, the Bulgarian ecotypes predominantly originate from ruderal and wild habitats up to 1360 m asl, with partly extreme climatic conditions such as average annual temperatures of 2.8 °C and low annual rainfall of 514 mm (Table 1). The low autumn and high spring growth might be a special adaptation to these collections sites. The collection from predominately ruderal and wild habitats might also explain the low plant biomass production of the Bulgarian ecotypes at the second cut in the second experimental year. The wide collection area with the diverse altitudes and climatic conditions is probably the reason for the high ecotypic variability regarding the observed traits, as indicated by the ANOVA and the PCA. Based on the broad diversity amongst the Bulgarian ecotypes, the accessions GR6562, GR6563, GR6601 and GR6609 were identified as best accessions, exceeding the average performance of the check varieties in respect to several traits. Accessions GR6562 and GR6563 were collected close to the city of Trigrad (province of Smoljan) in the Rhodope Mountains at 1140 and 1360 m asl from an orchard and from the roadside, respectively. Accessions GR6601 and GR6609 were collected in Sadovo (province Plovdiv) located in the Thracian Plain.

Crown rust (*Puccina coronata* f. sp. *lolii*) is the most severe infection in ryegrass as it reduces the herbage yield and the digestibility of dry matter and fastens senescence (Plummer et al. 1990; Kimbeng 1999). The incidence of rust symptoms, observed at the time of maximal infection, was medium to low in all trials, irrespective whether ecotypes or cultivars were considered. Obviously, the infestation pressure was moderate during the experimental period and differences between ecotypes and cultivars were not clearly pronounced. This is in accordance to findings of Kemesyte et al. (2010), while on the other hand several studies reported a more severe damage of ecotypes by rust infection than of cultivars (Oliveira-Prendes and Charmet 1989; Charmet et al. 1990; Charmet and Balfourier 1991). Interestingly, almost no rust symptoms were found for the ecotypes collected in Bulgaria (scores 1.4–2.2) in the material set examined. Probably no rust pathogens were present in this trial. However, the check varieties tested together with the Bulgarian ecotypes showed a slightly but clearly higher infection (scores 2.6–5.2). Corresponding to the results of this study, a low susceptibility to rust infection was also reported for perennial ryegrass ecotypes collected in the neighboring country Romania, particularly for populations from Crisana region, under field as well as laboratory conditions (Willner et al. 2010). The lower susceptibility of the Bulgarian ecotypes to rust infection in this study might be explained by the fact that they originated predominately from ruderal and wild habitats. Balfourier and Charmet (1991) reported a relation between the habitat at the place of origin and the susceptibility to rust infection. Populations collected from roadsides and paths were less susceptible to rust infection compared to populations collected from meadows. Furthermore, the low biomass production level of the Bulgarian ecotypes later in the year (PBM2 and GBW2) may have led to a low leaf area present for rust infection, and to climatic conditions in the sward which were not favorable for the spreading of the pathogen. This would also explain the correlation between plant biomass and rust susceptibility in our study (Table 7). Our assumption supports the findings of Balfourier and Charmet (1991), who reported that growth habit is in good relationship with rust susceptibility, and that prostrate populations are more susceptible to rust than erect ones. On the other hand, considering the different physiological races of *P. coronate* f. sp. *lolii*, which vary in their virulence to specific ryegrass genotypes (Kimbeng 1999), it might be that there was no compatible matching between avirulence genes of the local rust races and resistance genes of the ecotypes collected as described in the gene-for-gene concept of Flor (1971). Since the pathogen is able to rapidly adapt, virulent races might emerge when the Bulgarian ecotypes were grown for a longer period. However, similar to flowering tendency, aftermath heading and plant biomass, rust and disease resistance are results of a one year evaluation and further studies are necessary to validate these findings.

Conclusion

The collection of *L. perenne* ecotypes adapted to diverse environmental conditions and their thorough evaluation is an important step to identify interesting germplasm for specific breeding goals, as well as for future studies and research. Under the specific climatic conditions of the trial site at Malchow/Poel, the perennial ryegrass ecotypes collected in Bulgaria were predominantly characterized by a lower growth before winter (GBW1, GBW2), a rather low plant biomass development after the first cut (PBM2), but a high productivity in spring (GIS2, GIS3) and a low susceptibility to rust infection. Due to the large collection area and the diverse collection habitats, the Bulgarian ecotypes revealed a high variability concerning the ten traits evaluated, and several ecotypes could be identified which exceeded the average performance of the commercial cultivars. The accessions GR6562, GR6563, GR6601 and GR6609 exhibited a better performance in terms of spring growth in the second and third experimental year (GIS1, GIS2), growth before winter in the second year (GBW2) and rust infection (RUST2), and achieved comparable results regarding all the other traits investigated. The ecotypes collected in Ireland exhibited a low aftermath heading, showed an interesting performance regarding disease susceptibility and revealed well developed swards after winter in the third experimental year. The accessions GR7607, GR12044 and GR12053 were best developed after three years of cultivation, while accessions GR7600, GR7707, GR7724 were identified to combine a low susceptibility to rust and general diseases (virus, leafspot). Due to the relatively small collection area in the province of Galicia and the high share of ecotypes collected from old pastures, there was a low variability amongst the Spanish ecotypes which achieved a similar performance as the commercial check varieties. The material from Croatia was well comparable with the respective checks under the present experimental conditions and showed a high variation for the traits growth before winter in the first year, growth in spring and aftermath heading in the second year. In order to confirm these findings, the most promising accessions identified in our trials should be studied with more detail in the future in longer-term field experiments under varying environmental conditions.

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Authors' contributions SBP drafted the initial paper. EW designed and coordinated the field trials and evaluated the accessions in the field. EW and KJD contributed to the interpretation of the data and to the preparation of the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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